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Sibling rivalry versus mother's curse: can kin competition facilitate a response to selection on male mitochondria?

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Assuming that fathers never transmit mitochondrial DNA (mtDNA) to their offspring, mitochondrial mutations that affect male fitness are invisible to direct selection on males, leading to an accumulation of male-harming alleles in the mitochondrial genome (mother's curse). However, male phenotypes encoded by mtDNA can still undergo adaptation via kin selection provided that males interact with females carrying related mtDNA, such as their sisters. Here, using experiments with *Drosophila melanogaster* carrying standardized nuclear DNA but distinct mitochondrial DNA, we test whether the mitochondrial haplotype carried by interacting pairs of larvae affects survival to adulthood, as well as the fitness of the adults. Although mtDNA had no detectable direct or indirect genetic effect on larva-to-adult survival, the fitness of male and female adults was significantly affected by their own mtDNA and the mtDNA carried by their social partner in the larval stage. Thus, mtDNA mutations that alter the effect of male larvae on nearby female larvae (which often carry the same mutation, due to kinship) could theoretically respond to kin selection. We discuss the implications of our findings for the evolution of mitochondria and other maternally inherited endosymbionts.

1. Introduction

In most animal species, individuals inherit mitochondrial DNA (mtDNA) almost exclusively from their mothers [1]. This uniparental transmission creates an asymmetry in how mtDNA evolves in response to selection on males versus selection on females: direct selection on females can result in adaptation in the mitochondrial genome, while direct selection on males cannot [2–4]. As a result, theory predicts that mtDNA mutations that reduce male survival or reproductive output, but have no effect on females, can accumulate in the mitochondrial genome by genetic drift just as readily as neutral mutations. The predicted outcome is a male-biased mitochondrial mutation load termed mother's curse [2,3]. Furthermore, there is growing evidence that male-harming mutations that have pleiotropic beneficial effects on females may accumulate in the mitochondrial genome through positive selection [5–7].

Kin selection may provide a potentially widespread means by which mtDNA in males could undergo adaptation, even though males are effectively an evolutionary 'dead end' for mtDNA [8–13]. In short, kin selection might favour particular mtDNA haplotypes because of the phenotypes that they induce in males, provided that the male phenotype affects the fitness of non-randomly related females. For example, in a species in which females usually mate with their brothers, any mtDNA haplotype causing male infertility would be selected against because it harms the fitness of the male's sisters, who carry the same mtDNA [9–13]. The evolutionary response to kin selection on male mtDNA can be understood in terms of a modified version of Hamilton's rule,

$r_{mt}B - C > 0$ [14], which describes when a male phenotype encoded by mtDNA will be selectively favoured [9]. Here, r_{mt} denotes the correlation between the mtDNA of interacting males and females, where positive r_{mt} indicates that the interacting pair are more likely to share the same mitochondrial allele than a random male–female pair, and negative r_{mt} indicates a lower probability than the random expectation. B can be interpreted as the mitochondrial allele's indirect genetic effect (an 'mtIGE' [9]) on the fitness of the female recipient (or equivalently and more correctly, on the fitness of her mitochondria) and can be positive or negative. Finally, the cost C is the direct effect of the mutation on the fitness of the male's mitochondrial genome, which can be considered zero in species with complete uniparental inheritance because the mitochondria carried by males have no fitness left to lose. The inequality therefore reduces to $r_{mt}B > 0$, highlighting that male mtDNA evolution will occur when two conditions are satisfied: (i) interacting males and females must be non-randomly related with respect to their mitochondrial genomes ($r_{mt} \neq 0$), and (ii) variation in male mtDNA must covary with the fitness of (the mitochondria carried by) female social partners ($B \neq 0$).

To our knowledge, no study has yet explicitly measured r_{mt}, although r_{mt} is clearly non-zero in many commonly observed social interactions. For example, across many taxa, males interact with their female matrilineal siblings and their mother, for which $r_{mt} = 1$. Examples include altricial avian chicks sharing a nest, a group of seedlings growing in close proximity to each other and the maternal plant, or a brood of insect larvae that hatched from a clutch of eggs laid by a single female [15,16]. Under these scenarios, provided that the phenotype of males influences the fitness of their mother or sisters, kin selection might favour or disfavour particular mitochondrial haplotypes because of their effects on the male phenotype.

Although better studied than r_{mt}, the other key parameter B has only been measured in two previous studies, both of which focused on fitness effects of adult males on adult females arising through sexual interactions [9,17]. The goal of this study was therefore to measure the magnitude of B in a different social context, by testing whether the mtDNA carried by *Drosophila melanogaster* larvae influences the fitness of cohabiting larvae. Interactions between *D. melanogaster* siblings in the larval stage occur commonly in nature because adult females lay multiple eggs on single food resources [18,19], and because larvae actively seek each other out [20]. The same is true for many other insect taxa (e.g. Glanville fritillary butterflies, where larvae live on food plants in groups of roughly 100 full siblings [21]), meaning that the concepts we address here are likely to generalize beyond *Drosophila*. To maximize our chance of detecting this mitochondrial genetic effect, we setup dyads of one male and one female larva carrying a standardized nuclear genome and a known mtDNA haplotype (which we varied) and let them develop under food-limited conditions. This experimental design allowed us to test whether mtDNA had (i) a direct effect on the fitness of the individual carrying it and (ii) an indirect genetic effect on the fitness of a cohabiting opposite-sex individual (B). We measured two components of juvenile fitness: survival to adulthood and development time, as well as three selectively important adult traits (among those individuals that reached adulthood): body size [22,23], female offspring production and male reproductive competitive ability.

2. Methods

(a) Fly stocks

We maintained all fly stocks on a cornmeal, yeast and dextrose-based diet (electronic supplementary material, table S1 lists the full recipe) at 25°C under a 16 h light, 8 h dark cycle.

(b) Mitochondrial strains

To explore the effects of mtDNA variation on fitness, we used five lines of *Drosophila melanogaster* from a mitochondrial genotype reference panel (hereafter referred to as *mt*-strains [24–26]). These strains were sourced from populations around the world and each carries a distinct mitochondrial haplotype. The five *mt*-strains are named after the localities from which their mtDNA was sampled: Barcelona, Brownsville, Dahomey (now Benin), Israel and Sweden. To statistically partition mitochondrial and nuclear effects on phenotypic variance, Clancy [24] used a balancer crossing scheme to place each haplotype alongside a novel, standardized nuclear genome: the isogenic reference strain w^{1118}. At the time of our experiments, each *mt*-strain had been maintained for at least 70 generations by backcrossing *mt*-strain females to w^{1118} males. The w^{1118} line was itself kept isogenic by propagating it through a single full-sibling pair every generation. Furthermore, each of the five mitochondrial genomes has been cultured in two duplicate populations since 2007 (as described in [5]). Half of our experimental replicates used the five *mt*-strains that were (following previous studies presenting data on this mitochondrial panel) named 'Duplicate one', and half used the five strains named 'Duplicate two'. The duplicate *mt*-strains allow us to statistically separate the effects of the five mitochondrial haplotypes from other confounding differences between the ten individual *mt*-strains (e.g. residual differences in their nuclear genome, epigenome, microbiome or differences in environmental conditions brought about by vial-sharing). Furthermore, to ensure all cytoplasmic genetic effects were limited to the mitochondria, all *mt*-strains were cured of infection by the intracellular bacterium *Wolbachia* many generations prior to our experiments. Although our *mt*-strains use mitochondrial haplotypes from around the world, mitochondrial genetic variance is known to routinely segregate within natural populations of *D. melanogaster* [27–30].

(c) $w^{1118\text{-}GFP}$ stock

To differentiate between male and female embryos, we created a line expressing the transgenic construct GFP-tagged Sex lethal protein (*Sxl-GFP*), originally created by Thompson [31] and inserted on chromosome three. Using seven generations of backcrossing, we integrated *Sxl-GFP* into the w^{1118} genetic background, creating a stock we term $w^{1118\text{-}GFP}$ (electronic supplementary material, figure S1). *Sex lethal* is part of the sex determination pathway in *D. melanogaster*; it is expressed in the early embryo and has female-limited expression. Female embryos carrying the *Sxl-GFP* construct fluoresce green under UV light while male embryos do not, allowing eggs to be sorted by sex from approximately 6 h post-laying. The *Sxl-GFP* construct carries a copy of the *mini-white* reporter gene, which restores red-eye colour in flies carrying a defective allele of the *white* gene (as is found in w^{1118}). We exploited *mini-white* as a marker in order to differentiate progeny from different fathers in our assays of adult male fitness.

(d) Experimental mitochondrial strains

To produce the *mt*-strain flies used in our experiments, we crossed 3- to 5-day-old virgin females from each *mt*-strain to 3- to 5-day-old $w^{1118\text{-}GFP}$ males, creating lines heterozygous for the *Sxl-GFP* construct and nearby sites on chromosome three, but otherwise largely homozygous across their w^{1118} nuclear genome. Crucially,

these experimental *mt*-strain flies carried mtDNA inherited from their *mt*-strain mothers (electronic supplementary material, figure S1). As the experimental *mt*-strains inherited a single copy of the *mini-white* transgene linked to the *Sxl-GFP* construct, and because w^{1118} carries a mutant allele of *white*, the experimental *mt*-strain flies had orange eyes.

(e) *bw* 'standard' stock

To provide a standardized source of visually identifiable flies to compete and mate with our focal experimental flies in adult fitness assays, we used an inbred stock carrying a recessive mutation of *brown* (*bw*) that encodes a brown-eye colour phenotype (sourced from a teaching laboratory at the University of Melbourne). All experimental *bw* flies were the progeny of 3- to 5-day-old parents and were raised at a density of 100 larvae per vial. At the onset of adult fitness assays, all *bw* flies were 2 to 4 days old.

(f) Measuring mitochondrial genetic effects on juvenile viability

To explore direct and indirect mtDNA effects during larval development, we reared dyads of larvae, each containing one male and one female from specific *mt*-strains (see below) and measured components of their fitness. We conducted the experiment under resource-limited conditions to increase the likelihood that the health of one individual would affect the fitness of the other. As described above, we created progeny heterozygous for the *Sxl-GFP* construct, which also carried the maternally transmitted mtDNA haplotype of interest, by crossing females from each of the duplicate *mt*-strains with males from the $w^{1118\text{-}GFP}$ line (electronic supplementary material, figure S1). We then allowed females to oviposit on a grape juice agar plate for 3 h, then waited a further 6 h before sex-sorting the embryos under a Leica M165 FC Fluorescence microscope. In total, 17 individuals were incorrectly sexed from a total of 930 flies that survived to adult eclosion (98.2% accuracy). Misidentified flies and their social partners were removed from all subsequent analysis. To confirm we had not mistaken non-viable eggs for males, we waited 24 h to allow the sex-sorted larvae to hatch before allocating larvae to specific competing dyads.

To promote strong competition between larvae, and to simplify measurement of the effect of one individual's mtDNA on another's phenotype, we raised larvae in pairs in small containers of food. Specifically, we placed a pair of recently hatched first-instar larvae (one male, one female) into a 200 µl pipette tip containing approximately 8 µl of food medium, with a small piece of Blu Tack (Bostik SA, Australia) sealing the posterior opening, and a ball of cotton wool sealing the anterior opening. We used a factorial experimental design such that individuals from each of the five experimental *mt*-strains competed with a member of the opposite sex carrying every possible mitochondrial haplotype, giving 25 male–female combinations for each replicate. We never mixed flies from Duplicate one and two, to limit the size of the experimental design matrix (our design matrix had 50 cells—2 duplicates × 25 pairs of mtDNA haplotypes—while a full factorial design would have 100 cells). To prevent desiccation of the food, we placed the pipette tips in a tip rack inside a plastic container filled with a small amount of water and wrapped in parafilm. Once larvae reached third instar and began wandering, we removed them from the pipette tip and placed them in individual wells of a 96-well plate, where they subsequently pupated. We collected data over six experimental blocks, each of which contained 6–8 complete replicates of each cell of the experimental design (i.e. 6–8 × 50 pairs of larvae per block). Each replicate was made up of strains from one of the two duplicate strain groups, such that half the replicates contained *mt*-strains from Duplicate one and remaining replicates contained strains from Duplicate two.

We recorded egg-to-adult viability, to investigate whether sex, the focal individual's mtDNA and the mtDNA of the co-habiting larva (the 'social partner') affected survival in response to larval competition. For 751 of the 896 larvae that eclosed into adults, we measured egg-to-adult development time to the nearest hour, using time-lapse photography (under red light illumination during the dark phase of the light cycle); a technical issue with the video recording prevented measurement of development time in the remaining 145 larvae.

(g) Measuring mitochondrial genetic effects on adult fitness traits

(i) Body size

In blocks three to six, we recorded the wing length of 405 adult flies as a measure of body size. We removed the right wing of adult flies using forceps and mounted them on white cardboard using double-sided tape. If the right wing was damaged or missing, we instead used the left wing. We photographed each wing using a Leica M165 FC fluorescence microscope (8.3 × magnification) and scored wing length from the digital images using ImageJ software (NIH), by taking the distance from the anterior cross-vein to the end of the second longitudinal vein (following [32]).

(h) Female productivity

To measure female offspring production, we paired individual 3- to 5-day-old adult females from the larval competition phase of the experiment with one 2- to 4-day-old *bw* male. Each pair was housed in a food vial containing *ad libitum* yeast for 48 h, giving time for mating, behavioural interactions and oviposition. We then removed the adult flies and left the vial for 13 days to allow offspring to reach adulthood. Eclosed adult offspring were frozen, and the number of male and female offspring subsequently counted as a measure of female fitness.

(i) Male reproductive fitness

To measure male fitness, we counted the proportion of progeny sired by *mt*-strain males relative to a standard *bw* competitor. This fitness measure encompasses pre- and post-copulatory competition, as well as the survival rate of *mt*-strain males' offspring relative to those of standard *bw* males. We placed 3- to 5-day-old *mt*-strain males in a vial containing food medium with *ad libitum* access to yeast. In addition, we introduced a male–female pair of 3-day-old (± 1 day) virgin *bw* flies. These trios were left to interact—allowing for competition between the males and potentially also their sperm—for 3 days, after which the *bw* female was transferred to a fresh vial to oviposit for 48 h. Both the initial competition vial and the second vial for each female were left for 13 days as above. To measure the competitive ability of the orange-eyed *mt*-strain males, we counted the number of offspring with a red-eye (*mt*-strain male progeny) versus brown-eye phenotype (*bw* male progeny), summed across both of the female's vials.

(j) Statistical analysis

To test for an effect of mtDNA on traits expressed in both sexes (i.e. egg-to-adult viability, egg-to-adult development time and wing length), we used mixed models fitted using maximum likelihood (rather than restricted maximum likelihood) and implemented via the lmer or glmer functions from the lme4 and lmerTest packages for R (version 3.6.2). For each trait measurement, we began by defining a 'full model' containing a set of fixed factors that captured the most complex biological scenario that we considered plausible. The full model included the following fixed effects: (i) the focal individual's sex, (ii) its mitochondrial haplotype, (iii) the haplotype of its social competitor and (iv) all the

two- and three-way interactions between factors 1–3; additionally, the full model (and all other models examined) contained 'experimental block', 'duplicate strain' and 'dyad ID' as random effects. Dyad ID groups individual flies that developed together in the same pipette tip. We specified a binomial distribution and a logit link function for the egg-to-adult viability model and used a normal distribution for the egg-to-adult development time. Finally, we fitted a linear mixed model with an identity link function to assess the effects of mtDNA on wing length.

The female productivity data contained an excess of zeroes, so we analysed the number of offspring produced by females using a hurdle model with negative binomial errors, fitted with the glmmTMB package [33]. The hurdle model allowed us to measure the influence of the predictor variables on (i) the proportion of females that failed to produce any offspring and (ii) the number of offspring produced among those females that did produce offspring. For male reproductive fitness, we used a beta-binomial generalized linear model, in which the response variable was the identity of the father of the focal offspring (i.e. the focal male or the competitor *bw* male). To account for repeated measures of each pair of focal and rival males, we included male ID as a random effect. We removed data points in which the *bw* female produced no offspring, because such cases preclude measurement of the two males' relative mating/siring successes. To fit the models for the female- and male-limited fitness measures, we included focal haplotype, social haplotype and their interaction as fixed effects, and the random effects duplicate strain and block.

We determined the best fitting model for each response variable by ranking the full model against all possible simpler models (including the intercept-only model) by their AICc scores (the corrected Akaike information criterion; implemented via the dredge function from the R package MuMIn; [34]). Model evaluation results for each fitness component are presented in electronic supplementary material, tables S1–S8. As we commonly observed no clearly superior top model when ranking the models by AICc, we calculated model-averaged coefficients and their 95% confidence intervals based on the subset of models with ΔAICc < 6. This can be interpreted as averaging coefficients over all the models with an AICc value within six of the top model. As this was a planned experiment, we report the conditional average coefficients rather than the full average coefficients (following [35]).

3. Results

(a) Mitochondrial genetic effects on juvenile fitness

Overall, we assessed the fitness of 2056 larvae, across 44 replicates of each complete set of *mt*-strain cell combinations. 896 of 2056 (44%) larvae included in our analysis survived to adulthood. The predictors sex (RVI = 0.38, where RVI = relative variable importance and can be interpreted as the likelihood the fixed effect is present in the best performing model from the initial full set of possible models), focal haplotype (RVI = 0.23) and social haplotype (RVI = 0.07) were present in the set of models with ΔAICc < 6 (electronic supplementary material, table S2) that were used for model averaging. Neither mtDNA nor sex had a statistically significant effect on egg-to-adult viability (electronic supplementary material, table S3).

We measured egg-to-adult development time for 751 of the 896 flies that reached adulthood. Average development time was 2.17 h longer for males than females (electronic supplementary material, table S5; 95% CIs = 0.427 to 3.919, p = 0.015, RVI for sex = 0.87). Focal haplotype and social haplotype were retained in the averaged model subset, but with low probabilities of being included in the top model (RVI = 0.12 and RVI = 0.05; electronic supplementary material, table S4). There was some indication (albeit non-significant) that individuals carrying the Dahomey haplotype reached adulthood earlier than those carrying the Barcelona haplotype (averaged model estimate = −2.688, 95% CIs = −5.466 to 0.09, p = 0.058; electronic supplementary material, table S5).

(b) Mitochondrial genetic effects on adult fitness components

We measured wing length for 405 of the 896 eclosing flies. The model containing sex as the sole fixed effect provided a substantially better fit than all other models considered (electronic supplementary material, table S6); model averaging was therefore unnecessary. As expected, males were substantially smaller than females (averaged model estimate = −0.086 mm, 95% CIs = −0.1 mm to −0.072 mm, p < 0.001, RVI for sex = 1; electronic supplementary material, table S7). Neither focal nor social mtDNA haplotype had any detectable effect on body size.

We measured the offspring production of 424 females that reached adulthood, of which 291 produced offspring and 133 did not. Focal haplotype was retained in the averaged hurdle model but did not have any effect on whether a female was able to produce offspring (RVI = 0.09; figure 1*a*; table 1). However, we found evidence for an indirect genetic effect of male mtDNA on the ability of females to produce offspring (RVI for social haplotype = 0.89; electronic supplementary material, table S8). Females that shared a larval environment with males carrying the Israel mtDNA haplotype were significantly less likely to produce any offspring compared with females developing alongside males carrying the Barcelona haplotype (averaged model estimate = −1.051, 95% CIs = −0.395 to −1.707, p = 0.002; figure 1*b*; table 1). Among those females that produced at least one progeny, focal mtDNA haplotype (RVI = 0.74) had a significant effect on the number of progeny produced. Females that carried the Brownsville (averaged model estimate = −0.191, 95% CIs = −0.347 to −0.035, p = 0.016; figure 1*c*; table 1) and Sweden (averaged model estimate = −0.213, 95% CIs = −0.377 to −0.049, p = 0.011; figure 1*c*; table 1) haplotypes produced fewer offspring than females carrying the Barcelona haplotype. We detected no effect of social haplotype on the number of offspring produced among fertile females; it was not retained as a predictor in the averaged conditional component of the model.

In the assay of male reproductive fitness (n = 356), males carrying the Brownsville haplotype had close to zero fitness; they produced a significantly lower proportion of offspring than males carrying all other haplotypes (averaged model estimate = −2.614, 95% Cis = −3.552 to −1.676, p < 0.001; figure 2*a*; table 2). In addition to focal haplotype (RVI = 0.99), social haplotype (RVI = 0.09) was also retained as a predictor in the averaged model subset (electronic supplementary material, table S9). We found weak evidence (albeit non-significant) for an indirect genetic effect of mtDNA on reproductive fitness, although this result should be treated with caution given the low variable weight. Females carrying the Brownsville haplotype reduced the proportion of offspring produced by males with whom they shared a larval environment (averaged model estimate = −0.546, 95% CIs = −1.175 to 0.084, p = 0.089; figure 2*b*; table 2).

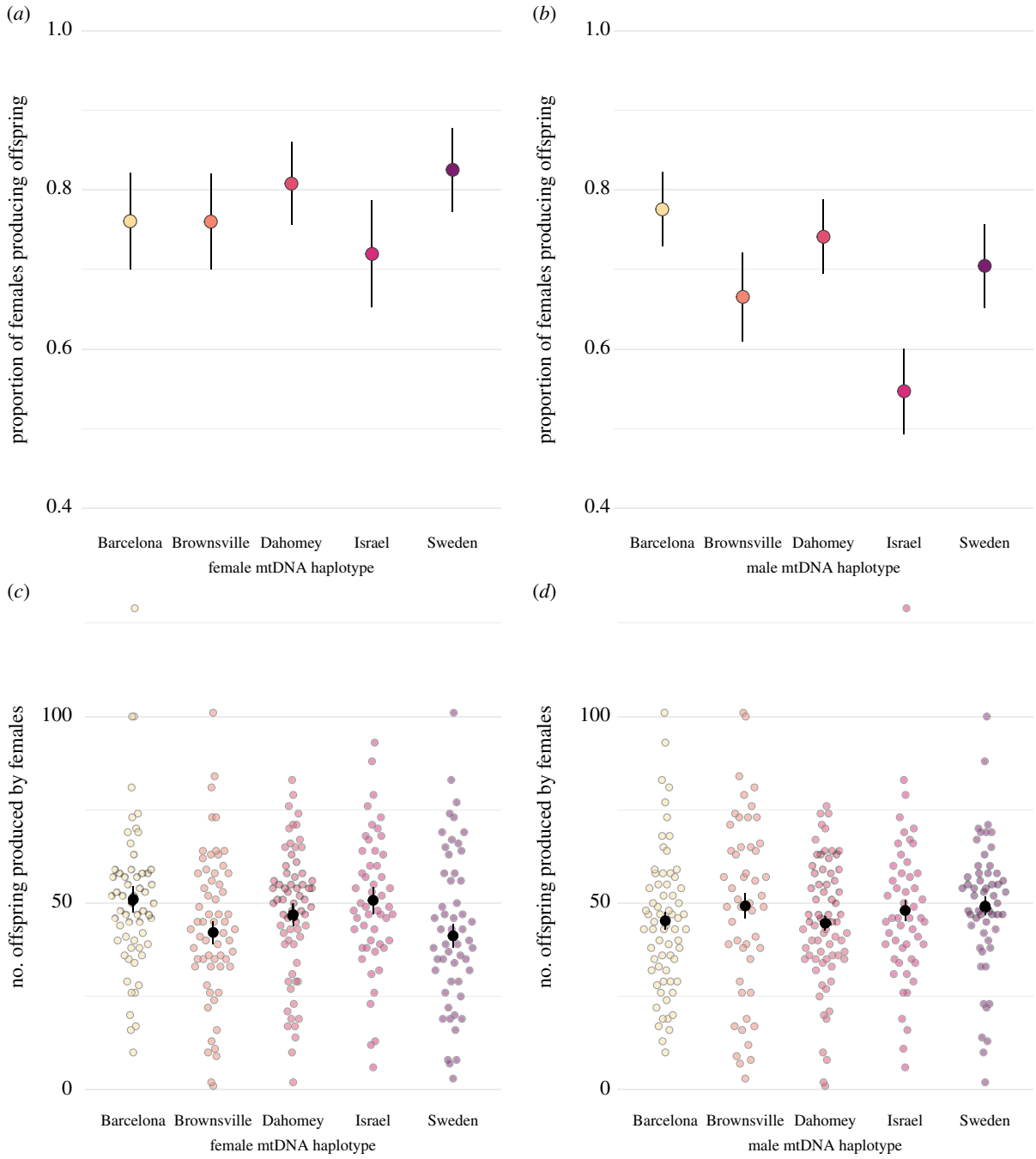

Figure 1. mtDNA directly and indirectly affects female fitness. (*a*,*b*) Model predictions of the mean proportion of females that produced offspring (the zero-inflated or hurdle component of the model) across (*a*) female focal mtDNA haplotypes and (*b*) social male mtDNA haplotypes. (*c*,*d*) Direct and indirect effects of mtDNA on the number of offspring produced by a female. Black points show model predictions of the mean for each haplotype in (*c*) and mean estimates from the raw data in (*d*), while coloured points represent offspring produced by individual females. Model predictions were not calculated for (*d*) because social haplotype was not retained as a predictor for the conditional component of the averaged hurdle model. Error bars depict standard errors in all plots. (Online version in colour.)

4. Discussion

We found evidence that mitochondrial genetic variance in *D. melanogaster* covaries with an individual's adult fitness, and the adult fitness of a social partner with whom they competed during larval development. Specifically, females carrying different mtDNA varied in their reproductive output, mtDNA haplotype directly affected male reproductive competitive ability, and male mtDNA haplotype indirectly affected the reproductive output of females with which the male developed as a larva. We found no evidence for direct or indirect genetic effects of mtDNA on larval viability, development time or body size (contra [5,36]).

Our results reaffirm the work of previous studies illustrating that mtDNA has non-neutral direct effects on phenotype [37]; mtDNA directly affected female productivity [5,38] and male reproductive fitness (reviewed in [39]). The Brownsville haplotype appeared to reduce fitness in adults of both sexes, confirming the low fitness of males carrying Brownsville mtDNA observed in previous studies [5,40,41], which is largely attributable to defects in sperm production [42]. However, our results contradict some earlier findings that Brownsville mtDNA has comparatively high fitness when expressed in females [5,9,41]. This might be explained by trait-specific effects of the Brownsville haplotype on female fitness. Camus

Table 1. Effects of mtDNA on female reproductive output. Conditional estimates from model averaging the full generalized linear mixed model are shown. Models were included in the averaging subset if delta <6. The zero-inflated component of the model relates to whether the female produced at least one offspring. The conditional component corresponds to the model fit after the hurdle was cleared, that is, how many offspring were produced by females that produced at least one offspring. Asterisks show significant effects. RVI = relative variable importance and can be interpreted as the likelihood the model term is present in the best performing model from the initial full set of possible models.

model term	RVI	model-averaged estimate	s.e.	2.5% CI	97.5% CI	*p*-value
zero-inflated component—probability of producing at least one offspring						
intercept		−1.205	0.285	−1.764	−0.645	< 0.001
focal haplotype	0.09					
Brownsville		0.03	0.324	−0.635	0.641	0.993
Dahomey		−0.28	0.332	−0.933	0.373	0.401
Israel		0.214	0.326	−0.427	0.855	0.513
Sweden		−0.396	0.363	−1.109	0.318	0.277
social haplotype	0.89					
Brownsville		0.552	0.358	−0.151	1.255	0.124
Dahomey		0.188	0.352	−0.504	0.88	0.595
Israel		1.051	0.334	0.395	1.707	0.002*
Sweden		0.371	0.360	−0.337	1.079	0.305
conditional component—females that produced at least one offspring						
intercept		3.911	0.076	3.762	4.059	< 0.001
focal haplotype	0.74					
Brownsville		−0.191	0.079	−0.347	−0.035	0.016*
Dahomey		−0.084	0.076	−0.234	0.065	0.27
Israel		−0.005	0.08	−0.163	0.152	0.947
Sweden		−0.213	0.083	−0.377	−0.049	0.011*

et al. [5] show that females carrying the Brownsville haplotype produce offspring with high egg-to-adult viability, but that the relative reproductive output of these females is highly dependent on age: young Brownsville females have high relative offspring production, while older females produce relatively few offspring. This contradiction might also be explained by environmental differences between study designs: we reared larvae in more stressful conditions than the previous studies (as evidenced by the low larval survival and adult body size observed in our experimental individuals, relative to flies reared in our laboratory using typical *Drosophila* culturing methods). It is possible that females carrying the Brownsville haplotype are particularly sensitive to stressful conditions during larval development. The extent to which mitochondrial genotypic effects on phenotype are moderated by differences in the external environment remain poorly studied, but emerging evidence suggests that mitochondrial genome by environment interactions may be extensive [43–46].

In addition to direct effects of mtDNA on phenotype, we also found evidence that mtDNA has an indirect genetic effect on the fitness of interacting social competitors. Males carrying the Israel haplotype appeared to negatively affect the ability of their female larval competitors to produce offspring and there was some indication that female larva carrying the Brownsville haplotype reduced the reproductive competitive ability of male larval competitors. This result illustrates that mitochondrial indirect genetic effects can occur in the context of juvenile development; that is, $B \neq 0$ in the modified version of Hamilton's rule discussed in the Introduction. Thus, the effects of mtDNA on the phenotype of male larvae can lead to the evolution of male phenotypes that are selectively advantageous for females, given that male and female larvae carrying the same mtDNA probably interact frequently in nature (i.e. $r_{mt} \neq 0$).

Our results suggest that kin selection may favour mitochondrial haplotypes that encode male-harming phenotypes when brothers compete with their sisters for resources. Reduced foraging efficiency in males may be one such adaptation, where female relatives benefit from reduced social competition [8]. Conflict over resources could theoretically lead to a more dramatic evolutionary response, including selection for mtDNA mutations that cause early-life mortality in males, as has been discovered in other maternally transmitted cytoplasmic genetic elements [47]. For example, Hurst [47] documents cases of male killing induced by maternally inherited cytoplasmic agents, many of which are attributed to the bacterial genus *Spiroplasma* (more recent findings include [48–50]), with later evidence also implicating *Wolbachia* [51–53]. As we do here, Hurst invokes kin selection as the driver of such adaptation and uses models to show that male-killing genes can successfully invade a population given high values of vertical transmission and benefits to siblings when brothers die [47]. Despite the putative benefits to the male's surviving sisters, to our knowledge, there are no known mitochondrial haplotypes that cause early-life male lethality. However, mtDNA can induce male-limited fertility impairment—a similarly extreme fitness effect [42,54,55]. It is possible that mitochondrial haplotypes that incur early-life male lethality may remain undiscovered in species where

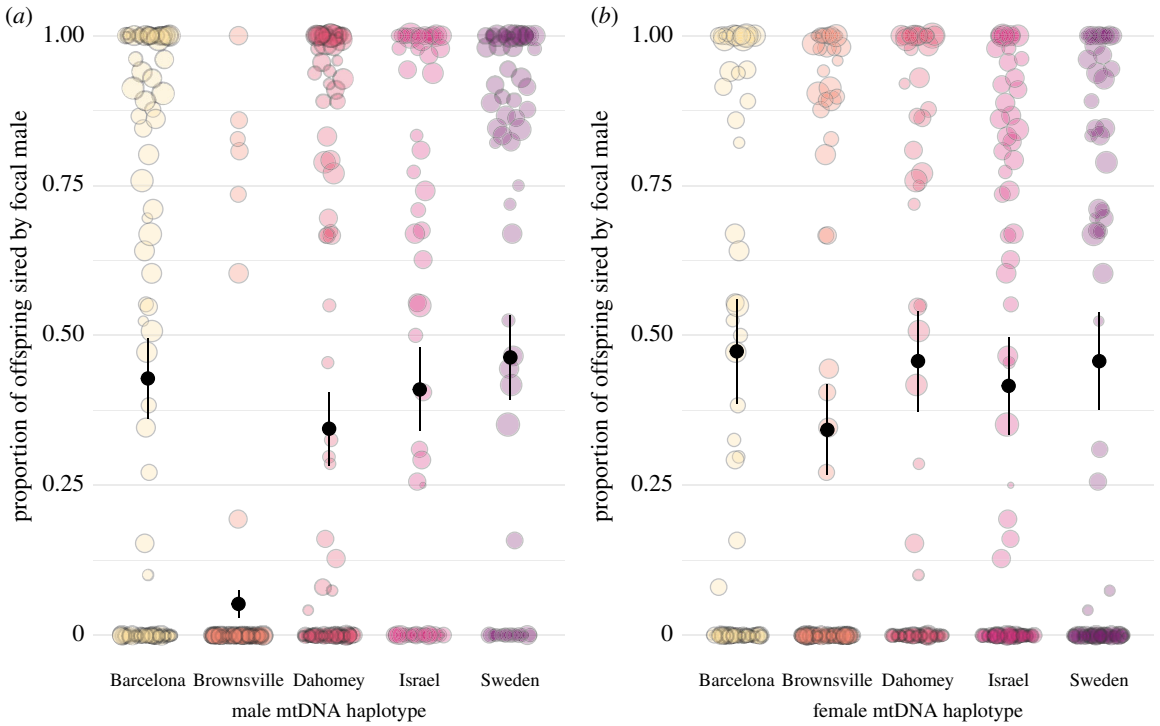

Figure 2. The proportion of offspring produced by *mt*-strain males competing with standard *bw* males. (*a*) Direct effect of mtDNA on male fitness. (*b*) Indirect genetic effect of female mtDNA on male fitness. Black points show model predictions of the mean proportion of offspring sired by the *mt*-strain male, with associated standard errors. Coloured points represent the proportion of offspring produced by individual *mt*-strain males, with larger points indicating a higher total number of offspring produced in the vial (sired by either male). (Online version in colour.)

Table 2. Effects of mtDNA on male reproductive fitness. Conditional estimates from model averaging the full generalized linear mixed model are shown. Models were included in the averaging subset if delta <6.

model term	RVI	model-averaged estimate	s.e.	2.5% CI	97.5% CI	*p*-value
intercept		−0.29	0.276	−0.83	0.251	0.294
focal haplotype	0.99					
Brownsville		−2.614	0.479	−3.552	−1.676	< 0.001*
Dahomey		−0.355	0.285	−0.914	0.205	0.214
Israel		−0.075	0.314	−0.691	0.54	0.81
Sweden		0.143	0.303	−0.452	0.737	0.638
social haplotype	0.09					
Brownsville		−0.546	0.321	−1.175	0.084	0.089
Dahomey		−0.065	0.326	−0.704	0.574	0.841
Israel		−0.233	0.316	−0.852	0.385	0.46
Sweden		−0.066	0.323	−0.699	0.568	0.839

sib–sib competition is a dominant force, or may lie cryptic as sterility effects have, after rescue via nuclear modifier alleles [56].

These hypothetical adaptations highlight that kin selection can theoretically exacerbate the effects of mother's curse, by selecting for male-harming phenotypes that benefit the males' female mitochondrial relatives. However, there are also circumstances where female fitness increases when male siblings are present and in good condition. For example, *Drosophila melanogaster* larvae feed for longer and on higher quality food in the presence of other larvae [20] and preferentially do so with related individuals [57]. Similarly, in the absence of parental care, burying beetle (*Nicrophorus vespilloides*) broods are selected to hatch synchronously, as large broods are better able to open and feed upon carcasses [58]. In circumstances such as these, where there is a threshold value of conspecific juveniles required to access a resource, or maximize foraging efficiency, siblings in the presence of other siblings will be favoured by selection. Such selection will reduce the male mutation load when mtDNA influences male viability but is constrained by equal action on female viability, as it does not matter if males or females survive, just as long as some individuals do. Kin selection will therefore most efficiently purge mutations with male-limited harmful effects on viability and condition when females specifically benefit from the presence and actions of

their brothers or their sons [10]. These conditions are likely to arise in socially complex vertebrates that undertake cooperative breeding, where mature offspring forego reproduction in favour of raising younger siblings [59]. One sex commonly helps more than the other in these systems; examples include cooperatively breeding bird species, where males generally help more than females [60], and certain mammal species, where males contribute to sibling fitness to a greater extent than females [61]. A response to selection on male mitochondrial variance in traits that affect offspring provisioning may therefore be possible in species that exhibit male-helping breeding systems.

In sum, we have shown that mitochondrial haplotypes in males have indirect genetic effects on females (and vice versa) during the larval development of *D. melanogaster*. Evidence for the existence of mitochondrial indirect genetic effects on fitness is increasing and has previously been demonstrated for interactions between adult insects in the genera *Callosobruchus* and *Drosophila* [9,17]. These effects may elicit an evolutionary response to selection on male mtDNA and affect the evolutionary trajectories of mitochondrial haplotypes within populations. We contend that a response to selection on male phenotypes can either reduce or increase male fitness, depending on whether male–female interactions are competitive or cooperative. While this study provides evidence that mtDNA effects on male phenotypes are very likely to respond to selection in certain circumstances, future work is required to definitively demonstrate male mtDNA evolution. We encourage the quantification of r_{mt} between interacting males and females in wild populations, and tests for mitochondrial indirect genetic effects of males on females in species other than well-studied insect laboratory models.

Data accessibility. All the raw data, supplementary figures and tables, and all R scripts can be found at https://tomkeaney.github.io/male_mtDNA_evolution/ and from the Dryad Digital Repository: https://doi.org/10.5061/dryad.np5hqbzq8 [62].

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